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Ploszaj et al., Amino acids (Austria), 2000, 19 (2), p483-96.

Stefanelli et al. Biochemical journal (England) May 1, 2000, 347 Pt. 3, p875-80.

Sakagami et al. Anticancer Research (Greece), Jan-Feb. 2000, 20 (1A), p265-70.

Ray et al., American journal of physiology, Cell physiology (US), Mar. 2000, 278 (3), pC480-9.

Bock et al. Radiation research (US), Dec. 1999, 152 (6), p604-10.

Dai et al. Cancer research (US), Oct. 1, 1999, 59 (19), p4944-54.

Bratton et al. Jo. of biological chemistry (US), Oct. 1, 1999, 274 (40), p28113-20.

Palyi et al. Anti-cancer drugs (England), Jan 1999, 10 (10, p103-11.

4 Li et al. Am. journal of physiology , April 1999, 276 (4 Pt. 1), pC946-54.

Ray et al. Am. journal of physiology, Mar. 1999, 276 (3 Pt. 1) pC684-91.

Das et al. Oncology Research (US), 1997, 9 (11-12), p565-72.

Monti et al.. Life Sciences (England), 1998, 62 (9), p799-806.

Lin et al., Experimental cell research, (US), Nov. 25, 1997.

Tome et al. biochemical Journal (England) Dec. 15, 1997, 328 (Pt. 3), p847-54.

Hu et al., Biochemical journal (England), Nov. 15, 1997, 328 (Pt. 1), p307-16.

Tome et al. biological signals (Switzerland), May -Jun 1997, 6 (3), p150-6.

Taguchi et al., Cell biochemistry and function (England), Mar 2001, 19 (1), p19-26.

Camon et al. neurotoxicology (US), Fall 1994, 15 (3), p759-63.

Shinki et al., Gastroenterology (US), Jan 1991, 100 (1), p113-22.

Heston et al. Prostrate (US), 1982, 3 (4), p383-9

Stefanelli et al, biochemical journal (England), Apr. 1, 2001. 355 (pt. 1), p199-206.

Lopez et al., biocell: official journal of the sociedades latinoamericanas de microscopia electronica... et. al. 9Argentina), Dec. 1999, 23 (3), p223-8.

Schipper et al. seminars in cancer biology (US), feb. 2000, 10 (1), p55-68.

Nilsson et al., biochemical journal (England) Mar. 15, 2000, 346 Pt. 3, p699-704.

giuseppina monti m. et al., biochemical and biophysical research commun. (US), Apr. 13, 1999, 257 (2), p460-5.

ratasirayakorn et al, j. of periodontology feb. 1999, 70 (2), p179-84

stabellini et al., Experimental and molecular parhology (US), 1997, 64 (3), p147-55.

Sparapani et al, experimental neurology (US), nov. 1997, 148 (1), p157-66..

Dhalluin et al., carcinogenesis (Eng.), Nov. 1997, 18 (11), p2217-23.

Inhibition of polyamine synthesis induces p53 gene expression but not apoptosis

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Li, Li, Ji Li, Jaladanki N. Rao, Minglin Li, Barbara L. Bass, and Jian-Ying Wang. Inhibition of polyamine synthesis induces p53 gene expression but not apoptosis. *Am. J. Physiol.* 276 (*Cell Physiol.* 45): C946–C954, 1999.—The nuclear phosphoprotein p53 acts as a transcription factor and is involved in growth inhibition and apoptosis. The present study was designed to examine the effect of decreasing cellular polyamines on p53 gene expression and apoptosis in small intestinal epithelial (IEC-6) cells. Cells were grown in DMEM containing 5% dialyzed fetal bovine serum in the presence or absence of α-difluoromethylornithine (DFMO), a specific inhibitor of polyamine biosynthesis, for 4, 6, and 12 days. The cellular polyamines putrescine, spermidine, and spermine in DFMO-treated cells decreased dramatically at 4 days and remained depleted thereafter. Polyamine depletion by DFMO was accompanied by a significant increase in expression of the p53 gene. The p53 mRNA levels increased 4 days after exposure to DFMO, and the maximum increases occurred at 6 and 12 days after exposure. Increased levels of p53 mRNA in DFMO-treated cells were paralleled by increases in p53 protein. Polyamines given together with DFMO completely prevented increased expression of the p53 gene. Increased expression of the p53 gene in DFMO-treated cells was associated with a significant increase in G_1 phase growth arrest. In contrast, no features of programmmed cell death were identified after polyamine depletion: no internucleosomal DNA fragmentation was observed, and no morphological features of apoptosis were evident in cells exposed to DFMO for 4, 6, and 12 days. These results indicate that *1*) decreasing cellular polyamines increases expression of the p53 gene and *2*) activation of p53 gene expression after polyamine depletion does not induce apoptosis in intestinal crypt cells. These findings suggest that increased expression of the p53 gene may play an important role in growth inhibition caused by polyamine depletion.

growth inhibition; proliferation; tumor suppressor gene; ornithine decarboxylase; IEC-6 cells

THE EPITHELIAL CELLS of the gastrointestinal mucosa are among the most rapidly proliferating cells in the body (14, 21). Normal structure and function of the mucosa depend on a regulated rate of division of proliferating cells in the mucous neck region in the stomach and the crypts in the small intestine (19, 21). Inhibition of intestinal mucosal growth occurs commonly in critical illness and leads to diarrhea, malabsorption, delayed healing, and impaired barrier function. Increasing evidence has indicated that the cellular polyamines spermidine and spermine and their precursor putrescine are necessary for normal mucosal growth and that decreasing cellular polyamine levels inhibit cell renewal (15, 20, 21, 33). Intracellular polyamine levels are highly regulated and primarily dependent on the activation or inhibition of ornithine decarboxylase (ODC), which catalyzes the first step in polyamine biosynthesis (13, 26). Our previous studies have shown that depletion of cellular polyamines by inhibition of ODC with α-difluoromethylornithine (DFMO) significantly decreases mucosal growth in vivo (34) as well as in vitro (36), but the exact mechanism by which polyamine depletion results in growth inhibition remains to be demonstrated.

Cell homeostasis is regulated by a balance among proliferation, growth arrest, and apoptosis. The recognition that negative growth control must be elucidated to comprehend the mechanisms by which appropriate cell numbers are maintained has attracted considerable interest. The p53 gene encodes for a nuclear phosphoprotein, which acts as a transcription factor and has been shown to be involved in the processes of growth inhibition and apoptosis (6, 10, 18, 22). The p53 protein is present in low concentrations in normal cells and is a negative factor for cell cycle control, since progression from the G_1 to the S phase is often blocked in cells expressing high levels of this protein. Induction of p53 expression by transfection with a conditional p53 expression vector, for example, inhibits cell cycle progression in a glioblastoma tumor cell line (22). When growth-arrested cells are stimulated to proliferate, induction of p53 expression inhibits progression from the G_0/G_1 to the S phase (18, 22).

Apoptosis is an energy-dependent and highly regulated process by which cells die without releasing their contents and without eliciting inflammation (17, 28). Apoptosis is absolutely required for the natural development and homeostasis of tissues in complex multicellular organisms (40). As such, it is likely that apoptosis is implicated in the regulation of normal growth in the gastrointestinal mucosa. It has been shown that stimulation of expression of the p53 gene induces growth arrest and/or apoptosis in a number of cell types (17, 29). The p53 protein can simultaneously induce the genetic programs of G_1 phase growth arrest and apoptosis within the same cell type in which apoptosis can proceed in G_1-arrested or cycling cells (17).

To our knowledge, there are no studies concerning changes in p53 gene expression and apoptosis after polyamine depletion in intestinal epithelial cells. Given that polyamine depletion abolishes intestinal epithelial cell growth, we investigate the mechanism of this growth arrest and the possible role of p53. The immediate goal of the present study was to determine whether

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decreasing cellular polyamine levels by treatment with DFMO induces expression of the p53 gene and apoptosis in cultured normal rat small intestinal crypt (IEC-6) cells.

MATERIALS AND METHODS

Chemicals and supplies. Disposable culture ware was purchased from Corning Glass Works (Corning, NY). Tissue culture media and dialyzed fetal bovine serum (FBS) were purchased from GIBCO (Grand Island, NY), and biochemicals were obtained from Sigma Chemical (St. Louis, MO). The DNA probes used in these experiments included pCR™II containing a mouse p53 gene cDNA (Invitrogen, San Diego, CA), pBR322 containing a human genomic fragment coding for retinoblastoma susceptibility (*Rb*) gene [no. 57450, American Type Culture Collection (ATCC)], and pHcGAP containing human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (no. 57090, ATCC). The p53 probe from the mouse is well characterized and has been used routinely in studies of p53 gene expression in the rat; homology between the species is >75%. The antibody against rat p53 protein was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The [α-^{32}P]dCTP (3,000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). DFMO was the kind gift of the Merrell Dow Research Institute (Cincinnati, OH).

Cell culture and general experimental protocol. The IEC-6 cell line was purchased from ATCC at *passage 13*. The cell line was derived from normal rat intestine and was developed and characterized by Quaroni et al. (27). IEC-6 cells originated from intestinal crypt cells as judged by morphological and immunological criteria. They are nontumorigenic and retain the undifferentiated character of epithelial stem cells.

Stock cells were maintained in T-150 flasks in DMEM supplemented with 5% heat-inactivated FBS, insulin (10 μg/ml), and gentamicin sulfate (50 μg/ml). Flasks were incubated at 37°C in a humidified atmosphere of 90% air-10% CO_2. Stock cells were subcultured once a week at 1:20; medium was changed three times weekly. The cells were restarted from original frozen stock every seven passages. Tests for mycoplasma were routinely negative. *Passages 15–20* were used in the experiments. There were no significant changes of biological function and characterization from *passage 15* to *20*.

In the first series of studies we examined whether depletion of cellular polyamines by DFMO could alter expression of the p53 gene in IEC-6 cells. The general protocol of the experiments and the methods were similar to those described previously (36). Briefly, IEC-6 cells were plated at 6.25×10^4 cells/cm^2 and grown in control cultures or cultures containing 5 mM DFMO or DFMO plus spermidine (5 μM) for 4, 6, and 12 days. The dishes were placed on ice, the monolayers were washed three times with ice-cold Dulbecco's PBS (D-PBS), and then different solutions were added according to the assays to be conducted.

In the second series of studies we examined whether increased expression of the p53 gene after polyamine depletion was associated with programmed cell death in IEC-6 cells. Changes in internucleosomal DNA fragmentation, the morphological features of apoptosis, and the distribution of cell cycle were measured at various times after exposure to DFMO with or without spermidine.

RNA isolation and Northern blot analysis. Total RNA was extracted with guanidinium isothiocyanate solution and purified by CsCl density gradient ultracentrifugation, as described by Chirgwin et al. (5). Briefly, the monolayer of cells was washed with D-PBS and lysed in 4 M guanidinium isothiocyanate. The lysates were brought to 2.4 M CsCl and centrifuged through a 5.7 M CsCl cushion at 150,000 *g* at 20°C for 24 h. After centrifugation the supernatant was aspirated and the tube was cut ~0.5 cm from the bottom with a flamed scalpel. The resulting RNA pellet was dissolved in Tris·HCl (pH 7.5) containing 1 mM EDTA, 5% sodium laurylsarcosine, and 5% phenol (added just before use). The purified RNA was precipitated from the aqueous phase by the addition of 0.1 vol of 3 M sodium acetate and 2.5 vol of ethanol in sequence. Final RNA was dissolved in water and estimated from its ultraviolet absorbance at 260 nm by using a conversion factor of 40 units. In most cases, 30 μg of total cellular RNA were denatured and fractionated electrophoretically by using a 1.2% agarose gel containing 3% formaldehyde and transferred by blotting to nitrocellulose filters. Blots were prehybridized for 24 h at 42°C with 5× Denhardt's solution and 5× standard saline sperm DNA. cDNA probes for p53, *Rb*, and GAPDH were labeled with [α-^{32}P]dCTP by using a standard nick translation procedure. Hybridization was carried out overnight at 42°C in the same solution containing 10% dextran sulfate and ^{32}P-labeled DNA probes. Blots were

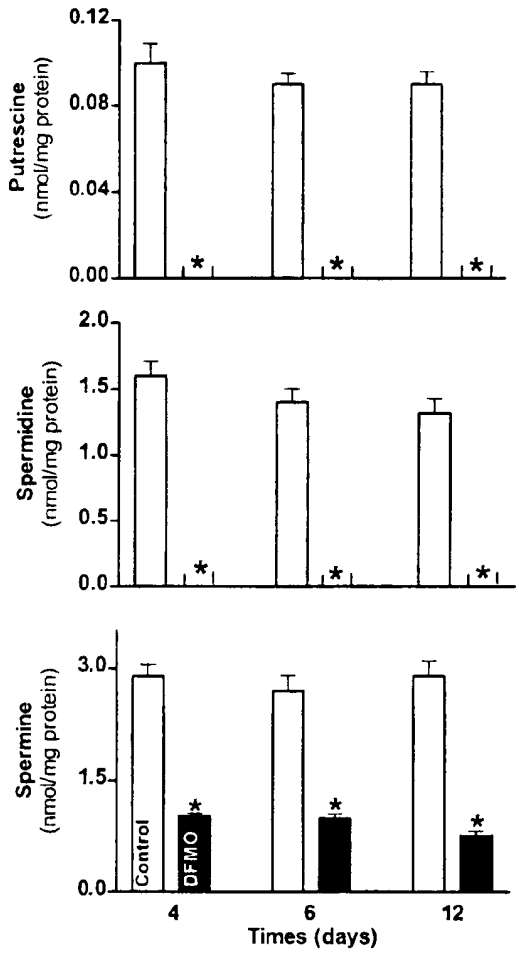

Fig. 1. Intracellular polyamine concentrations in IEC-6 cells grown in presence or absence of 5 mM α-difluoromethylornithine (DFMO) for 4, 6, and 12 days. Cells were grown in DMEM containing 5% dialyzed fetal serum. Medium was changed every 2nd day, and cellular polyamine levels were determined by HPLC analysis. Values are means ± SE from 6 dishes. * $P < 0.05$ compared with control groups.

washed with two changes of 1× saline sodium citrate-0.1% SDS at room temperature. After the final wash, the filters were autoradiographed with intensifying screens at −70°C. The signals were quantitated by densitometry analysis of the autoradiograms.

Western blot analysis of p53 protein. Cell samples, dissolved in SDS sample buffer, were sonicated for 20 s and centrifuged at 2,000 rpm for 15 min. The supernatant was boiled for 10 min and then subjected to electrophoresis on 7.5% acrylamide gels according to Laemmli (16). Each lane was loaded with 20 μg of protein equivalents. Briefly, after the transfer of protein onto nitrocellulose filters, the filters were incubated for 1 h in 5% nonfat dry milk in 1× PBS-Tween 20 (PBS-T) containing 15 mM NaH_2PO_4, 80 mM Na_2HPO_4, and 1.5 M NaCl, pH 7.5, and 0.5% (vol/vol) Tween 20. Immunological evaluation was then performed for 1 h in 1% BSA-PBS-T buffer containing 1 μg/ml monoclonal antibody against p53 protein. The filters were subsequently washed with 1× PBS-T and incubated for 1 h with goat anti-mouse IgG antibody conjugated to peroxidase. After extensive washing with 1× PBS-T, the immunocomplexes on the filters were reacted for 1 min with chemiluminescence reagent (NEL-100, Du Pont NEN). Finally, the filters were placed in a plastic sheet protector and exposed to autoradiography film for 30 or 60 s.

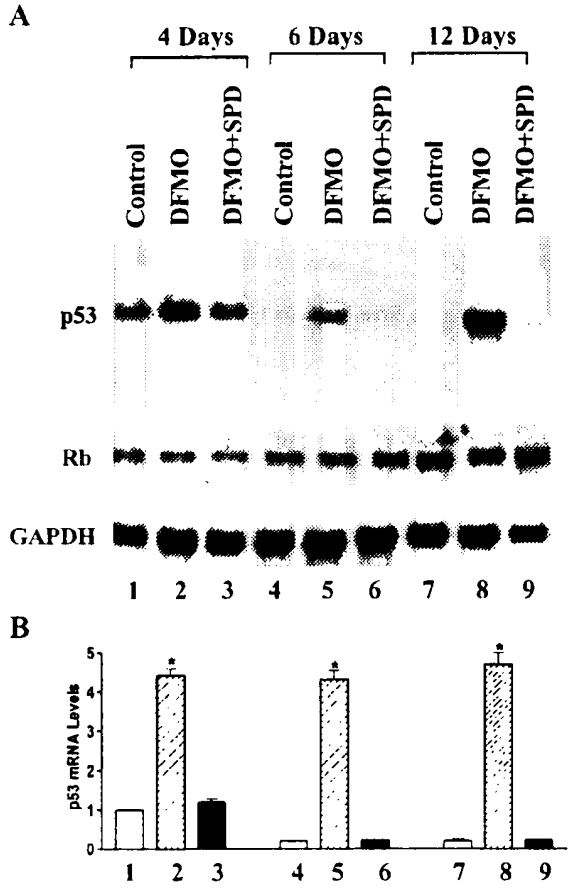

Fig. 2. Levels of p53 and retinoblastoma (*Rb*) mRNAs in control cells and cells treated with 5 mM DFMO or DFMO + spermidine (SPD). *A*: representative autoradiograms from control cells and cells exposed to DFMO or DFMO + spermidine for 4, 6, and 12 days. Total cellular RNA was isolated and examined by Northern blot analysis by using p53 and *Rb* gene cDNA probes at various days after initial plating. Hybridization to labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe served as a marker for equal loading of lanes. *B*: quantitative analysis of Northern blots by densitometry from cells described in *A*. Relative levels of mRNA for p53 were corrected for loading as measured by densitometry of GAPDH. Values are means ± SE of data from 3 separate experiments. * $P < 0.05$ compared with controls.

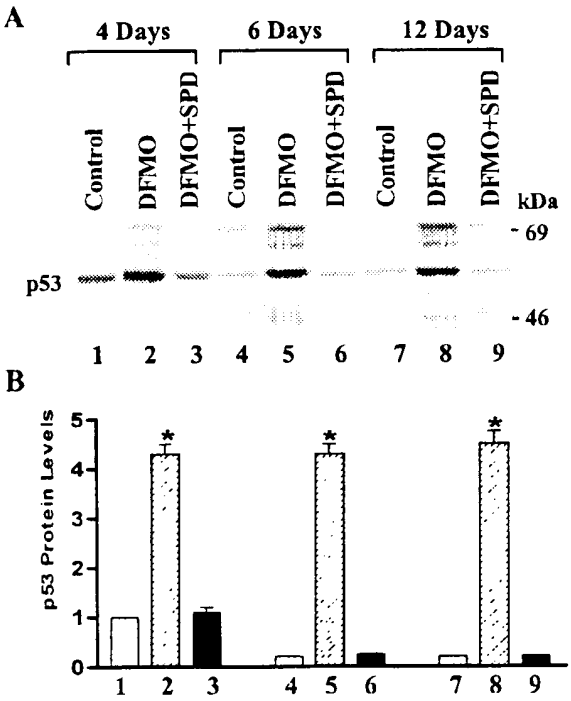

Fig. 3. Western blot analysis of p53 protein in extracts from cells described in Fig. 2. *A*: representative autoradiograms from cells exposed to DFMO or DFMO + spermidine for 4, 6, and 12 days. Protein (20 μg) was applied to each lane and subjected to electrophoresis on 7.5% acrylamide gel and Western immunoblotting. p53 protein was identified by probing nitrocellulose with a specific antibody described in MATERIALS AND METHODS, and position is indicated by p53. *B*: quantitative analysis of Western blots by densitometry from cells described in *A*. Values are means ± SE of data from 3 separate experiments. * $P < 0.05$ compared with control groups.

Immunohistochemical staining. Immunohistochemical staining for p53 protein was performed in IEC-6 cells by the indirect immunoperoxidase method, as described previously (12). Cells were plated at 6.25×10^4 cells/cm² on 22 × 22-mm glass coverslips, which were placed in 35-mm dishes in medium consisting of DMEM, 5% dialyzed FBS, 10 μg/ml insulin, and 50 μg/ml gentamicin sulfate. DFMO (5 mM) with or without 5 μM spermidine was added as treatment. At 6 days after initial plating, the cells were washed with D-PBS and then with D-PBS without Ca^{2+} and Mg^{2+} and fixed for 5 min at room temperature in 4% paraformaldehyde diluted with D-PBS. The cells were postfixed for 5 min with ice-cold methanol, rehydrated in D-PBS without Ca^{2+} and Mg^{2+} for 30 min at room temperature, and then incubated with goat polyclonal IgG raised against p53 protein at a dilution of 1:50 in humidified chambers for 24 h at 4°C. Nonspecific slides were incubated without antibody to p53 protein. The bound antibody was visualized with biotinylated anti-goat IgG and avidin-biotin complexes. The slides were counterstained with hematoxylin, mounted, and viewed with an Olympus microscope.

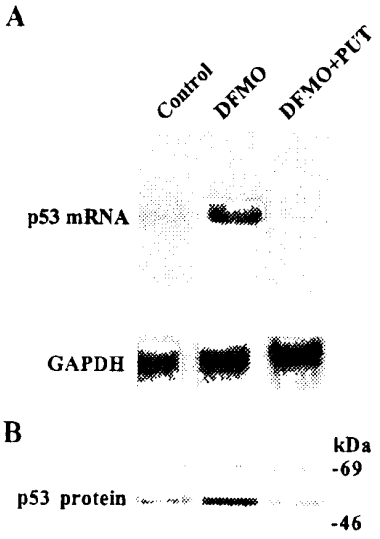

Fig. 4. Effects of DFMO and putrescine (PUT) + DFMO on expression of p53 gene in IEC-6 cells. *A*: representative autoradiograms from control cells and cells exposed to DFMO or DFMO + PUT for 6 days. p53 mRNA level was determined by Northern blot analysis. Loading of RNA was monitored by hybridization to labeled GAPDH probe. *B*: p53 protein measured by Western blot analysis from cells described in *A*. Three experiments were performed, and results were similar.

Determination of internucleosomal DNA cleavage. Internucleosomal DNA fragmentation was assayed by a modification of previously described methods (1). After cells were grown in the presence of DFMO with or without spermidine for various times, they were harvested and washed twice with cold PBS at 4°C. Cells were suspended in lysis solution containing 5 mM Tris·HCl, 20 mM EDTA, and 5% (vol/vol) Triton X-100 for 20 min on ice. The remaining steps for DNA fragmentation analysis were performed exactly as described previously (1). DNA samples were analyzed by electrophoresis in a 1.5% agarose slab gel containing 0.2% μg/ml ethidium bromide and visualized under ultraviolet illumination.

Flow cytometry analysis for cell cycle distribution. Cell sample preparation and propidium iodide staining for flow cytometry analysis were performed according to the method described by Nicoletti and Cooper (23). Briefly, IEC-6 cells were cultured in 10-cm plates at 6.25×10^4 cells/cm^2 and treated with DFMO with or without spermidine. Cells were harvested by trypsinization, washed twice in D-PBS, and fixed in 70% ethanol diluted with D-PBS. Cells were incubated in D-PBS containing RNase (100 μg/ml) and propidium iodide (40 μg/ml) at 37°C for 1 h before flow cytometry analysis. Cell cycle distribution was determined using a Coulter Epics V instrument with an argon laser set to excite at 488 nm. The results were analyzed using Elite 4.0 software (Phoenix Flow System).

Polyamine analysis. The nuclear polyamine content was analyzed by HPLC, as described previously (36). After washing the monolayers three times with ice-cold D-PBS, we added 0.5 M perchloric acid and then froze the monolayers at −80°C until ready for extraction, dansylation, and HPLC. The standard curve encompassed 0.31–10 μM. Values that fell >25% below the curve were considered not detectable. Protein was determined by the Bradford method (2). The results are expressed as nanomoles of polyamines per milligram of protein.

Statistics. Values are means ± SE from six dishes. Autoradiographic results were repeated three times. The significance of the difference between means was determined by ANOVA. The level of significance was determined using Duncan's multiple range test (11).

RESULTS

Effect of polyamine depletion on expression of the p53 gene. Administration of 5 mM DFMO, which totally inhibited ODC activity (36, 39), almost completely depleted cellular polyamines in IEC-6 cells (Fig. 1). The levels of putrescine and spermidine were undetectable at 4, 6, and 12 days after DFMO treatment. Spermine was less sensitive to the inhibition of ODC but was decreased by >60% in cells exposed to DFMO for 4, 6, and 12 days.

Inhibition of polyamine synthesis in the DFMO-treated IEC-6 cells was associated with a significant increase in expression of the p53 gene (Fig. 2). In control cells, steady-state levels of p53 mRNA were present at 4 days and then almost completely disap-

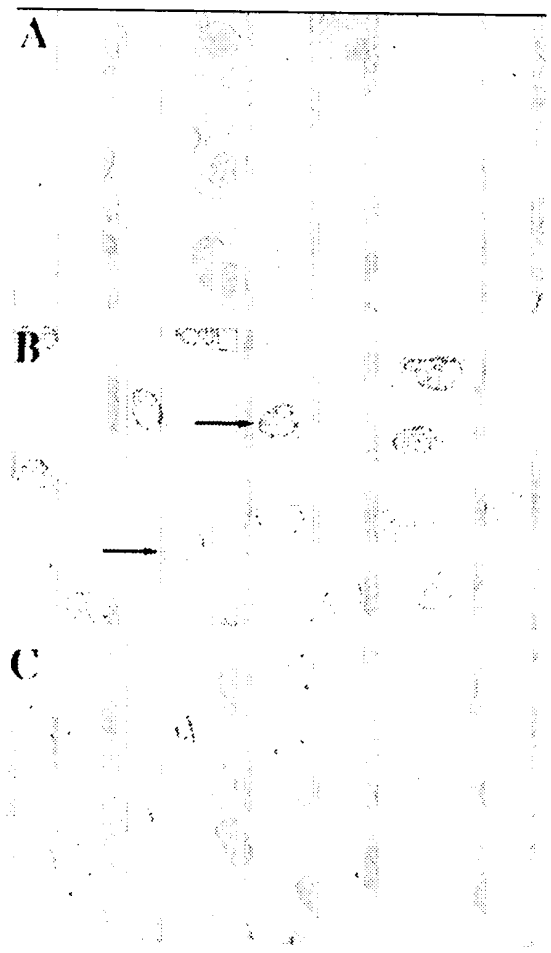

Fig. 5. Distribution of p53 protein in IEC-6 cells from all 3 treatment groups on *day 6*. Cells were fixed, permeabilized, and incubated with anti-p53 antibody used for Western blot analysis. Bound antibody was visualized with biotinylated anti-goat IgG and avidin-biotin complexes. *A*: control. *B*: cells treated with DFMO. *C*: cells treated with DFMO + spermidine. Arrows, p53-positive nuclei.

peared at 6 days, with decreased expression even at 12 days after plating. Depletion of cellular polyamines by treatment with 5 mM DFMO significantly increased expression of the p53 gene (Fig. 2). The increase in mRNA levels for the p53 gene was noted at 4 days and remained elevated at 12 days after exposure to DFMO. Maximum increases in p53 mRNA levels occurred between 6 and 12 days after addition of DFMO and were >10 times control values. Spermidine (5 μM) given together with DFMO completely prevented the increased expression of the p53 gene. The concentrations of p53 mRNA in cells treated with DFMO plus spermidine were indistinguishable from those in cells grown in control cultures. To investigate the specificity of this polyamine effect on p53, *Rb* gene expression was measured. In contrast to p53, polyamine depletion did not induce expression of the *Rb* gene in IEC-6 cells. There were no significant changes in *Rb* mRNA levels between control cells and cells exposed to DFMO with or without spermidine (Fig. 2).

Increased levels of p53 mRNA in cells exposed to DFMO were paralleled by increases in p53 protein (Fig. 3). Increases in p53 protein levels in cells exposed to DFMO for 6 and 12 days were ~ 10 times control values. The p53 protein concentration was returned to normal levels when DFMO was given together with spermidine. Putrescine at 10 μM had an effect equal to spermidine on the expression of the p53 gene when it was added to cultures that contained DFMO (Fig. 4).

To extend the positive findings of induced expression of the p53 gene after polyamine depletion, we further examined the cellular distribution of p53 protein in IEC-6 cells by immunohistochemical staining techniques. In control cells, no significant immunostaining for p53 protein was observed 6 days after initial plating (Fig. 5*A*). Consistent with our data from Northern and Western blot analysis, the nuclear immunoreactivities for p53 protein increased dramatically after polyamine depletion by treatment with DFMO (Fig. 5*B*). Increased p53 immunostaining was visible just inside the nuclei. Spermidine given together with DFMO prevented the increased immunostaining for p53 protein. The appearance of p53 in the cells treated with DFMO plus spermidine was the same as in control cells (Fig. 5*C*).

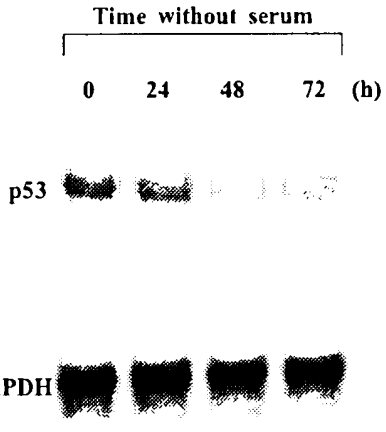

Fig. 6. Effect of serum starvation on expression of p53 gene in IEC-6 cells. Cells were grown in standard growth medium for 48 h and deprived of serum for 24, 48, and 72 h. Total cellular RNA was harvested and measured by Northern blot analysis with use of a p53 gene cDNA probe. Loading of RNA was monitored by hybridization to labeled GAPDH probe. Three experiments were performed, and results were similar.

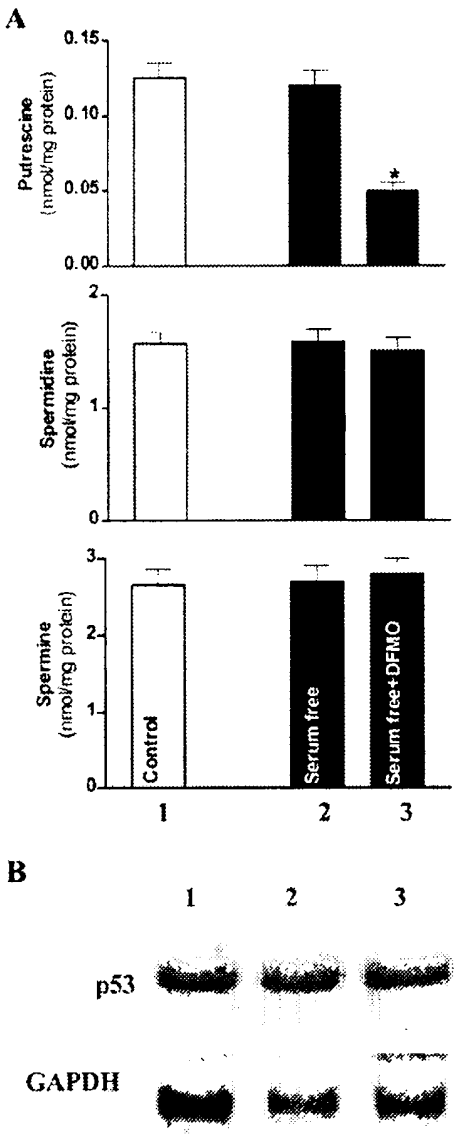

Fig. 7. Intracellular polyamines (*A*) and p53 mRNA levels (*B*) in IEC-6 cells subjected to serum starvation and treated with DFMO for 24 h. Cells were grown in standard growth medium for 48 h and then subjected to serum starvation and treated with 5 mM DFMO for 24 h. Cellular polyamine levels were determined by HPLC analysis, and p53 mRNA level was measured by Northern blot analysis with use of a p53 gene cDNA probe. Loading of RNA was monitored by hybridization to labeled GAPDH probe. Values are means ± SE of data from 6 dishes. $^*P < 0.05$ compared with controls.

Effect of serum starvation on cellular polyamines and p53 gene expression. To test the specificity of increased expression of the p53 gene after polyamine depletion,

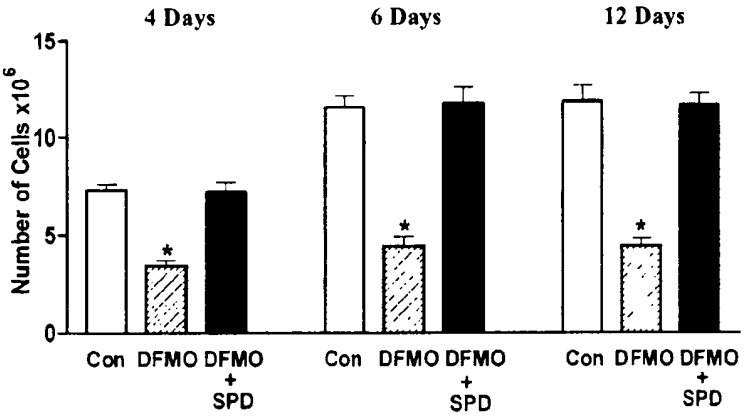

Fig. 8. Growth of IEC-6 cells described in Fig. 1. Cells were exposed to DFMO or DFMO + spermidine, and number of cells was determined at 4, 6, and 12 days. Values are means ± SE of data from 6 dishes. * $P < 0.05$ compared with controls.

we examined the effect of serum starvation on expression of the p53 gene. Our previous study demonstrated that starvation of IEC-6 cells by the removal of serum for 72 h decreased DNA synthesis by >75% (data not shown). In this study it has been shown that there was no significant decrease in cellular polyamines when cells were grown in the absence of serum for 72 h (data not shown). Consistent with the effect on cellular polyamines, serum-deprived quiescent IEC-6 cells were not associated with an increased expression of the p53 gene (Fig. 6). In fact, p53 mRNA levels slightly decreased at 48 and 72 h after serum deprivation. These results clearly show that serum-deprived quiescent IEC-6 cells do not decrease cellular polyamines and therefore have no effect on p53 gene expression. These findings suggest that increased expression of the p53 gene in the DFMO-treated cells is specifically related to polyamine depletion and does not result simply from decreased growth.

Fig. 9. Analysis of internucleosomal DNA fragmentation in IEC-6 cells exposed to DFMO or DFMO + spermidine. Cells were grown in presence of DFMO with or without spermidine for 6 days, and fragmented DNAs were extracted. DNA samples were analyzed by electrophoresis in a 1.5% agarose slab gel containing 0.1% ethidium bromide. Three experiments were performed, and results were similar.

We have also determined the changes in cellular polyamines and p53 gene expression in cells subjected to serum starvation and treated with DFMO (5 mM) for 24 h. Although putrescine content was significantly decreased, exposure to DFMO without serum for 24 h did not decrease spermidine and spermine levels (Fig. 7*A*). There were no differences of p53 mRNA levels between cells subjected to serum starvation alone and cells subjected to serum starvation and treated with DFMO (Fig. 7*B*). These results suggest that p53 gene expression is modulated by the cellular polyamines spermidine and spermine but not by their precursor putrescine. The diamine putrescine must then be converted to spermidine and spermine to regulate p53 gene expression in intestinal epithelial cells.

Changes in cell proliferation and apoptosis after polyamine depletion. Figure 8 shows that increased expression of the p53 gene in the DFMO-treated IEC-6 cells was associated with a significant decrease in cell numbers. With the activation of p53 gene expression after polyamine depletion, cell numbers were significantly decreased at 4 days, an effect maintained for up to 12 days. In the presence of DFMO, increased p53 gene expression and decreased cell numbers were completely prevented by addition of exogenous spermidine. The level of p53 protein (Figs. 3 and 5) and the number of cells were indistinguishable in cells exposed to DFMO plus spermidine and control cells.

Because the increased expression of the p53 gene has been shown to induce apoptosis in a number of cell types, studies were carried out to determine the involvement of apoptosis in the polyamine-deficient IEC-6 cells. We measured changes in internucleosomal DNA fragmentation and morphological features of apoptosis in cells exposed to DFMO or DFMO plus spermidine for 4, 6, and 12 days. No features of apoptosis were identified: no internucleosomal DNA fragmentation was observed (Fig. 9), and no typical morphological features of programmed cell death were evident in cells treated with DFMO with or without spermidine (Fig. 5). There were no apparent changes in cell viability in DFMO-treated or control cells.

Cell cycle analysis demonstrated that ~40% of IEC-6 cells accumulated in the G_1 phase at 4 days after initial plating in the control group (without DFMO; Fig. 10*A*).

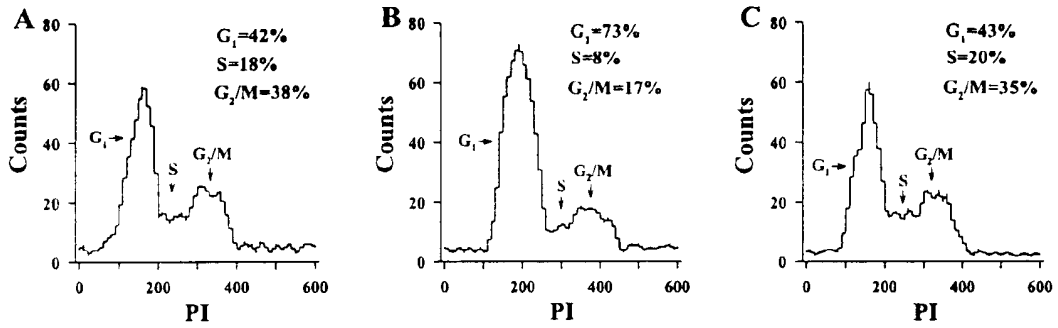

Fig. 10. Flow cytometric analysis of cell cycle distribution in IEC-6 cells. Cells were treated with DFMO or DFMO + spermidine for 4 days, then they were harvested and stained with propidium iodide (PI) and analyzed by flow cytometry. Percentages of cells in G_1, S, G_2/M phases of cell cycle are indicated. Three experiments were performed, and results were similar.

In polyamine-deficient cells, G_1 cells significantly increased to >70% (Fig. 10*B*), along with decreased populations in the S and G_2/M phases. There was not a hypodiploid peak (diagnostic of apoptotic cell death) in the DFMO-treated cells, further evidence that polyamine depletion did not induce spontaneous cell death. Cell cycle distribution in the cells exposed to DFMO plus spermidine was identical to that in control cells (Fig. 10*C*). We also measured cell cycle distribution at 6 days after initial plating and demonstrated that G_1 cells increased ~20% in control cells and cells exposed to DFMO plus spermidine (data not shown). We believe that the magnitude of change is diminished because the cells are entering the plateau phase of growth as they near confluence. The results in cells exposed to DFMO for 6 days were similar to those observed after treatment for 4 days.

DISCUSSION

Cell proliferation in the intestinal mucosa is dependent on the supply of polyamines to the dividing cells in the crypts (20, 21, 30, 35). Decreasing cellular polyamines significantly inhibits cell renewal, but the mechanism involved in growth inhibition remains to be elucidated. In the present study we investigated the effect of inhibition of polyamine biosynthesis on p53 gene expression and apoptosis in intestinal epithelial cells. Our results clearly show that depletion of cellular polyamines by treatment with DFMO significantly increases p53 mRNA levels, which were paralleled by increases in p53 protein (Figs. 2 and 3). The activation of p53 gene expression after polyamine depletion is associated with a significant increase in G_1 phase growth arrest but without apoptosis (Figs. 9 and 10). These findings suggest that expression of the p53 gene is highly regulated by cellular polyamines and may play an important role in the process of mucosal growth inhibition after polyamine depletion.

Although exact roles for cellular polyamines in specific biochemical events related to cell proliferation at the molecular level are largely unknown, several studies have indicated that expression of the protooncogenes c-*fos*, c-*myc*, and c-*jun* is at least partially involved in the early modulation of mucosal growth stimulation by polyamines (36, 37). Induction of mucosal growth in vivo (31) as well as in vitro (36) is accompanied by a significant increase in protooncogene expression after an increase in cellular polyamines, which precedes the induction of DNA synthesis. Polyamines are required for the protooncogene transcription, and depletion of cellular polyamines prevents increases in protooncogene expression and cell proliferation (25). However, polyamine-deficient cells have been shown to continuously maintain a high basal level of protooncogene expression (25, 36), indicating that growth inhibition after polyamine depletion must result from a mechanism other than a simple decrease in protooncogene expression. The change in activation of protooncogene expression is mainly relevant to the process of growth stimulation by polyamines but plays a minor role in growth inhibition after polyamine depletion (25, 36).

The recognition that negative growth control, including growth inhibition and programmed cell death, must be understood to comprehend how appropriate cell numbers are maintained in normal mucosa and how alterations in any part of the equation contributes to mucosal atrophy after polyamine depletion led us to consider the possibility that growth inhibition in DFMO-treated cells could be due in part to the activation of growth-inhibiting gene expression. The results reported here support our hypothesis and indicate that administration of DFMO not only completely depletes cellular polyamines but also significantly increases p53 gene expression in intestinal epithelial cells. Increased expression of the p53 gene in DFMO-treated cells is related to polyamine depletion rather than to a nonspecific effect of DFMO, because polyamine given together with DFMO prevents the increase in the levels of p53 mRNA and protein.

Three experiments were performed to further characterize the relationship between cellular polyamines and induced expression of the p53 gene in intestinal epithelial cells. First, we demonstrated that polyamine depletion has no effect on *Rb* gene expression (Fig. 2). Second, cellular polyamines are not decreased in serum-deprived quiescent IEC-6 cells and are not associated with the activation of p53 gene expression (Fig. 6).

Third, we compared polyamine-deficient cells with control cells in which growth was inhibited at confluence. As can be seen in Figs. 2 and 8, although depletion of polyamines by DFMO significantly increases p53 gene expression, there are no increases in p53 mRNA and protein levels in control cells in which growth was inhibited at confluence at 6 and 12 days after initial plating. These results indicate that polyamines have a specific effect on p53 gene expression and that increased levels of p53 mRNA and protein in DFMO-treated cells result from decreasing cellular polyamines and are not a secondary effect of growth inhibition.

To elucidate the biological significance of increased p53 protein after polyamine depletion, we examined the change in programmed cell death in DFMO-treated cells. Data presented in Figs. 9 and 10 show that depletion of cellular polyamines does not induce apoptosis. These results are consistent with the results of Casero et al. (3), which demonstrated that polyamine depletion alone did not result in programmed cell death in the human lung tumor cell line NCI H157. However, other studies indicate that the cellular polyamines are involved in the process of apoptosis. Decreased cellular polyamines and increased activity of the polyamine catabolic enzyme spermidine/spermine N^1-acetytransferase have been shown in dexamethasone- and polyamine analog-induced apoptosis (7, 9). An imbalance of polyamine metabolism may be a trigger of apoptosis in heat shock treatment- and γ-irradiation-induced cell death, in which increases in ODC mRNA and activity are observed without subsequent increases in cellular polyamine levels (8).

The nature of the molecular mechanisms that activate the expression of the p53 gene after polyamine depletion remains to be demonstrated. Although increased p53 protein is paralleled by a significant increase in p53 mRNA levels in DFMO-treated cells, it is not clear whether increased p53 mRNA is due to an increase in the gene transcription or results from the alteration of the mRNA stabilization. There is no doubt that cellular polyamines play different roles in the expression of various growth-related genes and that their effects are cell type dependent (36, 38). It has been shown that cellular polyamines are absolutely required for c-*myc* and c-*jun* mRNA synthesis in IEC-6 cells and that depletion of cellular polyamines by treatment with DFMO significantly decreases the transcription rates of these two genes but has no effect on their posttranscription (25). In contrast, polyamines negatively regulate the stability of transforming growth factor-β mRNA without affecting the gene transcription (24). Clearly, further studies are necessary to determine the transcriptional and posttranscriptional regulation of the p53 gene in IEC-6 cells after polyamine depletion.

In summary, these results indicate that inhibition of polyamine synthesis by treatment with DFMO significantly increases expression of the p53 gene in IEC-6 cells. Although the exact role of p53 protein in DFMO-treated cells is still unclear, our results clearly show that increased p53 gene expression after polyamine depletion does not induce programmed cell death. The association of induced p53 and a significant increase in G_1 phase growth arrest in DFMO-treated cells suggests that the activation of p53 gene expression may play an important role in the process by which polyamine depletion results in growth inhibition.

The authors thank Dr. Leonard R. Johnson (The University of Tennessee College of Medicine) for helpful discussion and Dr. Diane C. Fadely for critical reading of the manuscript.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-45314 and a Merit Review Grant from the Department of Veterans Affairs to J.-Y. Wang.

Some of these data have been published in abstract form (32).

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Received 11 August 1998; accepted in final form 6 January 1999.

REFERENCES

1. **Bhalla, K., and A. B. Pardee.** Taxol induces internucleosomal DNA fragmentation associated with programmed cell death in human myeloid leukemia cell. *Leukemia* 7: 563–568, 1993.
2. **Bradford, M. A.** A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of dying binding. *Anal. Biochem.* 72: 224–254, 1976.
3. **Casero, R. A., B. Go, H. W. Theiss, J. Smith, S. B. Baylin, and G. D. Luk.** Cytotoxic response of the relatively difluoromethylornithine-resistant human lung tumor cell line NCI H157 to the polyamine analogue N^1,N^8-bis(ethyl) spermidine. *Cancer Res.* 47: 3964–3967, 1987.
4. **Cheng, L., S. Zheng, K. Raghunathan, D. G. Priest, M. C. Willingham, J. S. Norris, and W. Fan.** Characterization of taxol-induced apoptosis and altered gene expression in human breast cancer cells. *Cell. Pharmacol* 2: 249–257, 1995.
5. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18: 5294–5299, 1979.
6. **Deppert, W.** The yin and yang of p53 in cellular proliferation. *Cancer Biol.* 5: 187–202, 1994.
7. **Desiderio, M. A., E. Grassilli, E. Bellesia, P. Salomoni, and C. Franceschi.** Involvement of ornithine decarboxylase and polyamines in glucocorticoid-induced apoptosis of rat thymocytes. *Cell Growth Differ.* 6: 505–513, 1995.
8. **Grassilli, E., M. A. Desiderio, E. Bellesia, P. Salomoni, F. Benatti, and C. Franceschi.** Is polyamine decrease a common feature of apoptosis? Evidence from gamma rays- and heat shock-induced cell death. *Biochem. Biophys. Res. Commun.* 216: 708–714, 1995.
9. **Ha, H. C., P. M. Woster, J. D. Yager, and R. A. Casero.** The role of polyamine catabolism in polyamine analogue-induced programmed cell death. *Proc. Natl. Acad. Sci. USA* 94: 11557–11562, 1997.
10. **Harris, C. C.** The p53 tumor suppressor gene: at the crossroads of molecular carcinogenesis, molecular epidemiology and cancer risk assessment. *Science* 262: 1980–1981, 1993.
11. **Harter, J. L.** Critical values for Duncan's new multiple range test. *Biometrics* 16: 671–685, 1960.
12. **Hsu, S. M., L. Raine, and H. Fanger.** Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques a comparison between ABC and unlabeled antibody (PAP) procedures. *J. Histochem. Cytochem.* 29: 577–580, 1981.
13. **Janne, J., L. Alhonen, and P. Leinonen.** Polyamines: from molecular biology to clinical applications. *Ann. Med.* 23: 241–259, 1991.
14. **Johnson, L. R.** Regulation of gastrointestinal mucosal growth. *Physiol. Rev.* 68: 456–502, 1988.
15. **Johnson, L. R., C.-C. Tseng, P. Wang, and M. K. Haddox.** Mucosal ornithine decarboxylase in the small intestine: localization and stimulation. *Am. J. Physiol.* 256 (*Gastrointest. Liver Physiol* 19): G624–G630, 1989.

16. **Laemmli, U. K.** Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685, 1990.
17. **Liebermann, D. A., B. Hoffman, and R. A. Steinman.** Molecular controls of growth arrest and apoptosis: p53-dependent and independent pathways. *Oncogene* 6: 199–210, 1995.
18. **Lin, D., M. T Shields, S. J. Ullrich, E. Appella, and W. E. Mercer.** Growth arrest induced by wild type p53 protein blocks cells prior to or near the restriction point in late G_1 phase. *Proc. Natl. Acad. Sci. USA* 89: 9210–9214, 1992.
19. **Lipkin, M., P. Sherlock, and B. Bell.** Cell proliferation kinetics in the gastrointestinal tract of man. II. Cell renewal in the stomach, ileum, colon, and rectum. *Gastroenterology* 45: 721–729, 1963.
20. **Luk, G. D., L. J. Marton, and S. B. Baylin.** Ornithine decarboxylase is important in intestinal mucosal maturation and recovery from injury in rats. *Science* 210: 195–198, 1980.
21. **McCormack, S. A., and L. R. Johnson.** Role of polyamines in gastrointestinal mucosal growth. *Am. J. Physiol.* 260 (*Gastrointest. Liver Physiol.* 23): G795–G806, 1991.
22. **Mercer, W. E., M. T. Shields, M. Amin, G. J. Sauve, E. Appella, J. W. Romano, and S. J. Ullrich.** Negative growth regulation in a glioblastoma tumor cell line that conditionally expresses human wild-type p53. *Proc. Natl. Acad. Sci. USA* 87: 6166–6170, 1990.
23. **Nicoletti, I., and T. Cooper.** A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J. Immunol. Methods* 139: 271–279, 1991.
24. **Patel, A. R., J. Li, B. L. Bass, and J.-Y. Wang.** Expression of the transforming growth factor-β gene during growth inhibition following polyamine depletion. *Am. J. Physiol.* 275 (*Cell Physiol.* 44): C590–C598, 1998.
25. **Patel, A. R., and J.-Y. Wang.** Polyamines modulate the transcription but not posttranscription of protooncogenes c-*myc* and c-*jun* in IEC-6 cells. *Am. J. Physiol.* 273 (*Cell Physiol.* 42): C1020–C1029, 1997.
26. **Pegg, A. E.** Polyamine metabolism and its importance in neoplastic growth and as a target for chemotherapy. *Cancer Res.* 48: 759–774, 1988.
27. **Quaroni, A., J. Wands, R. L. Trelstad, and K. J. Isselbacher.** Epithelial cell cultures from rat small intestine. *J. Cell Biol.* 80: 248–265, 1979.
28. **Steller, H.** Mechanisms and genes of cellular suicide. *Science* 267: 1445–1450, 1995.
29. **Vogelstein, B., and K. W. Kinzler.** p53 function and dysfunction. *Cell* 70: 523–526, 1992.
30. **Wang, J.-Y., and L. R. Johnson.** Polyamines and ornithine decarboxylase during repair of duodenal mucosa after stress damage in rats. *Gastroenterology* 100: 333–343, 1991.
31. **Wang, J.-Y., and L. R. Johnson.** Expression of proto oncogenes c-*fos* and c-*myc* in healing of gastric mucosal stress ulcers. *Am. J. Physiol.* 266 (*Gastrointest. Liver Physiol.* 29): G878–G886, 1994.
32. **Wang, J.-Y., J. Li, A. R. Patel, M.-L. Li, and B. L. Bass.** Polyamine depletion induces p53 gene expression but not apoptosis in IEC-6 cells (Abstract). *Gastroenterology* 11: A418, 1997.
33. **Wang, J.-Y., J. Li, A. R. Patel, S. Summers, L. Li, and B. L. Bass.** Synergistic induction of ornithine decarboxylase by asparagine and gut peptides in intestinal crypt cells. *Am. J. Physiol.* 274 (*Cell Physiol.* 43): C1476–C1484, 1998.
34. **Wang, J.-Y., S. A. McCormack, M. J. Viar, and L. R. Johnson.** Stimulation of proximal small intestinal mucosal growth by luminal polyamines. *Am. J. Physiol.* 261 (*Gastrointest. Liver Physiol.* 24): G504–G511, 1991.
35. **Wang, J.-Y., S. A. McCormack, M. J. Viar, and L. R. Johnson.** Secretin inhibits induction of ornithine decarboxylase activity by gastrin in duodenal mucosa and IEC-6 cells. *Am. J. Physiol.* 267 (*Gastrointest. Liver Physiol.* 30): G276–G284, 1994.
36. **Wang, J.-Y., S. A. McCormack, M. J. Viar, H. Wang, C.-C. Tzen, R. E. Scott, and L. R. Johnson.** Decreased expression of proto oncogenes c-*fos*, c-*myc*, and c-*jun* following polyamine depletion in IEC-6 cells. *Am. J. Physiol.* 265 (*Gastrointest. Liver Physiol* 28): G331–G338, 1993.
37. **Wang, J.-Y., and K. Takeuchi.** Polyamines in gastrointestinal mucosal cell proliferation. *J. Gastrointest. Res.* 4: 86–92, 1996.
38. **Wang, J.-Y., M. J. Viar, H.-J. Shi, A. R. Patel, and L. R. Johnson.** Differences in transglutaminase mRNA after polyamine depletion in two cell lines. *Am. J. Physiol.* 274 (*Cell Physiol* 43): C522–C530, 1998.
39. **Wang, J.-Y., H. Wang, and L. R. Johnson.** Gastrin stimulates expression of protooncogene c-*myc* through a process involving polyamines in IEC-6 cells. *Am. J. Physiol.* 269 (*Cell Physiol.* 38): C1474–C1481, 1995
40. **Wyllie, A. H., J. F. R. Kerr, and A. R. Currie.** Cell death: the significance of apoptosis. *Int. Rev. Cytol.* 68: 251–270, 1980.